



Stimulation of apical and basolateral vascular endothelial growth factor-A and vascular endothelial growth factor-C secretion by oxidative stress in polarized retinal pigment epithelial cells

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Purpose: To investigate whether oxidative stress modulates vascular endothelial growth factor (VEGF)-A and VEGF-C expression and polarized secretion in a human retinal pigment epithelium cell line (ARPE-19).

Methods: Long-term culture of ARPE-19 cells in Dulbecco's modified Eagle medium (DMEM)/F12 containing 1% fetal bovine serum (FBS) on transwell filters (12 mm or 6 mm, pore size 0.4 μ m) was performed to produce polarized retinal pigment epithelium (RPE) monolayers. The integrity of polarized monolayer was established by measurement of transepithelial resistance (TER) and presence of tight junctions assessed by zonula occludens (ZO-1) and occludin expression and apical Na/K ATPase localization. Paracellular permeability was studied using radiolabeled mannitol. Confluent cells were treated with tertiary butyl hydrogen peroxide (tBH) for varying durations (0-5 h) and doses (50-200 μ M). VEGF-A and -C expression was evaluated by western blot and quantitative RT-PCR, while secretion to the apical and basolateral surfaces was quantitated by ELISA.

Results: Polarity of ARPE-19 cells was verified by the localization of tight junction proteins, ZO-1 and its binding partner occludin by confocal microscopy as well as by localization of Na,K-ATPase at the apical surface. The TER in confluent ARPE-19 cells averaged $48.7 \pm 2.1 \Omega \cdot \text{cm}^2$ and tBH treatment (0-5 h) did not alter TER significantly ($46.9 \pm 1.9 \Omega \cdot \text{cm}^2$; $p > 0.05$ versus controls) or ZO-1 expression. Whole cell mRNA in nonpolarized ARPE-19 increased with tBH at 5 h both for VEGF-A and VEGF-C and the increase was significant ($p < 0.05$ vs controls). A similar, maximal increase at 5 h tBH treatment was also observed for VEGF-A and VEGF-C cellular protein levels. The secretion of VEGF-A and VEGF-C in nonpolarized ARPE showed an increase with tBH exposure. The levels of secretion of VEGF-A and -C were significantly higher in polarized monolayers and were stimulated significantly with tBH at both apical and basolateral domains. The secretion of VEGF-A increased with 150 μ M of tBH treatment as a function of time (1-5 h) with maximal increases at 5 h from 410 to 2080 pg/10⁶ cells on the apical and 290 to 1680 pg/10⁶ cells on basolateral domains. The pattern of VEGF-C secretion was similar. VEGF-A secretion was dose-dependent for the tBH range of 50-200 μ M and apical secretion tended to be higher than basolateral secretion.

Conclusions: Our data show that oxidative stress to RPE from tBH upregulates secretion of both VEGF-A and C. The secretion to the apical side was higher than that of basolateral side for VEGF-A and C. Given the role of VEGF in choroidal neovascularization, these data may be of value in understanding pathogenic mechanisms and designing antiangiogenic therapies.

Oxidative stress has been implicated as a possible inducer of choroidal neovascularization complicating age-related macular degeneration (AMD) which is mediated by upregulation of several angiogenic growth factors. In AMD, the retinal pigment epithelium (RPE) is an early site of injury, leading to visual loss [1]. The RPE performs critical visual maintenance functions including the degradation of shed photoreceptor outer segments and lipid peroxidation [2]. Lipid peroxidations trigger oxidative stress, generating high oxygen tension and reactive oxygen species (ROS), which in turn play a role in the progression to AMD [1].

Experimental evidence indicates that the RPE is a key player in the pathogenesis of choroidal neovascularization (CNV), which occurs late during the course of AMD [3,4]. In the course of CNV, RPE cells have been observed to encapsulate the new vessels, with their basal side toward the endothelial cells, which coincides with the arrest of further vascular growth [4], suggesting that RPE also has a function in controlling CNV. It has been well documented that ROS participate in decreased retinal blood circulation, increased vascular permeability and disruption of the outer blood-retinal barrier [5,6]. Polarized RPE in the intact monolayer as well as nonpolarized RPE that have migrated from the monolayer into the stroma promote CNV by secreting growth factors such as vascular endothelial growth factor (VEGF) [7]. Studies using oxidative stress-inducing agents showed excessive secretion of VEGF is associated with increased vascular permeability,

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disruption of blood-retinal barrier, and neovascularization in models of diabetic retinopathy [5,6,8].

In the human retina, the VEGF family of cytokines is crucial for its maintenance functions and its dysfunctions [9]. VEGF-A is a prototype of directly acting angiogenic factors, because it is secreted in a biologically active form and its receptors are found at sites of angiogenesis. In conjunction with several antiparallel disulfide-linked homodimeric isoforms (121, 145, 165, 189, 206 amino acids), five additional members (VEGF-B, C, D, E, and PlGF) of the VEGF family have been documented [10-12]. In resting RPE, VEGF-A is expressed at low levels, while under pathological conditions such as CNV, it triggers angiogenesis and increased vascular permeability. In many different cell systems, the upregulation of VEGF-A gene expression due to oxidants such as H_2O_2 and *t*-butylhydroperoxide (tBH), cyclic AMP (cAMP) analogs, tumor necrosis factor- α (TNF- α), transforming growth factor- β (TGF- β), cobalt, interleukin-1 β (IL-1 β) and hypoxia is well documented [13-16]. VEGF-A, when secreted, can function in an autocrine and a paracrine manner to regulate angiogenesis [15,17].

VEGF-C is one of the most potent directly acting lymphangiogenic factors belonging to the VEGF family [18]. It was first discovered and identified as a ligand of flt-4 (or VEGFR-3) expressed on lymphatic endothelium in adult skin and vascular tumors [19]. By activating fms-related tyrosine kinase 4 (flt-4), VEGF-C induces proliferation of lymphatic endothelial cells in vitro [19] and lymphangiogenesis in vivo [20]. Animal experimentation has shown that overexpression of VEGF-C in the skin of transgenic mice could result in proliferation and enlargement of lymphatic endothelium, but not of vascular endothelium. While most of the literature on VEGF-C relate to lymphangiogenesis, one report implicating a role for VEGF-C in ocular angiogenesis has appeared recently [21].

Epithelial cells possess a highly polarized structure, which helps them perform an array of functions, one of which is the polarized transport and secretion of proteins into apical or basolateral compartments [22]. The tight junction forms a partially occluding seal that retards diffusion of solutes across the paracellular space [23], and this barrier function of the tight junction enables the monolayer to establish and maintain concentration gradients between its apical and basal environments. Functional characteristics usually used to assess tight junction function barrier include electric resistance and charge and size of molecules permitted to cross the intercellular space [24]. The junction-specific protein, ZO-1 is a peripheral membrane protein bound on the cytoplasmic surface of junctional contacts and is expressed in all tight junctions [25].

Appropriate RPE function relies highly on the maintenance of its polarity. However, primary cultures generally fail to preserve many of the phenotypic characteristics, which are exhibited by RPE in vivo. So we selected ARPE-19, which forms polarized monolayers after prolonged culture on permeable support at low serum conditions [26]. The ARPE-19 model of polarized RPE has been used in studies of polarized

transport, polarized protein expression/secretion, [27,28] and barrier breakdown [29]. The apical side of the ARPE-19 cells represents the retina-facing domain of the RPE monolayer, whereas the basolateral side of the ARPE-19 cells represents the choroid-facing side of the monolayer.

The aim of this study was to determine the rates of secretion of VEGF-A and VEGF-C in polarized ARPE-19 cells and to examine whether oxidative stress causes selective changes in their vectorial secretion.

METHODS

Materials: ARPE-19 cells were obtained from American Type Culture Collection (Manassas, VA) and used between passages 22 and 25. Tissue culture-treated polyester transwells (6.5 mm or 12 mm in outer diameter and 0.4 μ m in pore size) were purchased from Costar (Corning, NY). Dulbecco's modified Eagle medium (DMEM) and Ham's F-12 medium were from Mediatech (Herndon, VA). Mouse laminin was from BD Biosciences (Bedford, MA). Other cell culture reagents and supplies were obtained from Life Technologies (Grand Island, NY).

Preparation of filter ARPE-19 monolayer cultures: Cultures of ARPE-19 cells were trypsinized and resuspended in DMEM/F12 supplemented with 100 U/mL penicillin-streptomycin, 2 mM L-glutamine, and 1% FBS. Approximately 1.7×10^5 cells/cm² were seeded in individual laminin-coated transwell filters in 0.5 ml (0.4 μ m pore size, 6.5 mm or 12 mm diameter). At the basal compartment, 1 ml medium was added, thereby leveling the height of the liquid to prevent hydrostatic pressure. The medium was changed twice a week. Cells were cultured for at least a month to form differentiated monolayers, with the apical domain corresponding to the retinal facing side of the RPE monolayer and the basolateral domain corresponding to the choroidal facing side of the RPE monolayer [26,27,30].

Measurement of transepithelial resistance: Transepithelial resistance (TER) of ARPE-19 monolayers on transwells was measured with an EVOM epithelial tissue voltammeter (World Precision Instruments, Sarasota, FL). All TER measurements were made in a cell culture hood within 3 min of removal of transwells from the incubator, and the average temperature at the time of measurement was 32.2 ± 1.85 °C. Net TER measurements were calculated by subtracting the value of a blank, laminin-coated filter without cells from the experimental value. Final resistance-area products ($\Omega \cdot \text{cm}^2$) were obtained by multiplication with the effective growth area. Measurements of TER were made once a week and on the day of the experiment. Confluent ARPE-19 cells grown in transwell filter inserts were treated with 150 μ M tBH for 1, 2, 3, 4, and 5 h and measurements of TER were made at hourly intervals. Cells in the medium that were not exposed to tBH served as controls. In each independent experiment, 3-6 filters were used per time point.

Characterization of tight junctions: The morphologic extent of polarization was visualized by immunolocalization of the tight-junction-associated proteins ZO-1 and occludin

and apical localization of Na/K ATPase. ARPE-19 monolayers grown on transwell filters were fixed in ice-cold methanol or 2% paraformaldehyde followed by three washes in phosphate buffered saline (PBS) and subsequently permeabilized with 0.1% Triton-X 100 for 30 min. Specimens were blocked in 5% rabbit or goat serum before incubating with ZO-1 rabbit polyclonal antibody in (1:100 dilution, Zymed, Carlsbad, CA), rabbit polyclonal anti-occludin (1:100, Zymed) and monoclonal antibody of anti-Na/K ATPase (1 µg/ml, Upstate, Lake Placid, NY) at 4 °C overnight. The cells were washed three times, and incubated with FITC conjugated anti-rabbit secondary antibody (Jackson Labs, West Grove, PA) for 1 h at room temperature. Cells were mounted with fluorescent medium containing DAPI (Vector Laboratories, Burlingame, CA) and viewed on an LSM 510 laser-scanning microscope (Carl Zeiss, Thornwood, NY). After the immunostaining procedure, membranes were removed from the inserts with a fine, sharp, sterile razor by inserting it at one side of the filter and then gently moving it around the filter.

Paracellular permeability of ARPE-19 cells: The permeability of the ARPE-19 monolayer was assessed by measuring the apical to basolateral and basolateral to apical movements of [3H] mannitol. The experiment was initiated by replacing the apical or basal medium with fresh medium containing 0.1 µM (1.7 uCi/ml) [3H] mannitol. One-tenth of the total volume of the medium at the receiving chamber was sampled for radioactivity quantification at different time intervals up to 3 h. Radioactivity was quantified by counting aliquots in a liquid scintillation counter. Paracellular permeability was measured by subtracting the contribution from laminin coated from the total permeability and is expressed as µl/cm²/min.

Quantitative real-time polymerase chain reaction: Total RNA was isolated from cells using TRIzol, (Invitrogen Life Technologies, Carlsbad, CA), and the contaminating genomic DNA was removed (DNA-free, Ambion, Austin, TX). Reverse transcription was performed with 1 µg RNA as per the manufacturer's protocol (Reverse Transcription System, Promega, Madison, WI). The PCR experiments were performed using SYBR green as the interaction agent (Roche Diagnostics, Indianapolis, IN). Each 20 µl PCR contained cDNA template, SYBR Green PCR master mix, and 0.5 µM each gene-specific primer. Quantification analysis of VEGF-A and VEGF-C mRNA was normalized using β-actin as reference. The specificity of PCR amplification products was checked by performing dissociation melting curve analysis. The sequences of primers used for VEGF-A were as follows: forward-5'-CTA CCT CCA CCA TGC CAA GTG-3', reverse-5'-TGC GCT GAT AGA CAT CCA TGA-3' and VEGF-C forward-5'-CTG CCG ATG CAT GTC TAA ACT G-3'; reverse-5'-TCT TGT TCG CTG CCT GAC ACT-3'. Relative multiples of change in mRNA expression was determined by calculation of 2^{-CT}. Results are reported as mean (±SEM) difference in relative multiples of change in mRNA expression.

Western blot analysis: Protein from ARPE-19 cells was extracted from cells using standard methods, and protein con-

tent was quantified with a protein assay (Bio-Rad, Richmond, CA,) with bovine serum albumin (BSA) standard. Equal amounts of protein were resolved on Tris-HCl polyacrylamide gels under reducing conditions (120 V, Ready Gel; Bio-Rad, Hercules, CA, USA) and transferred to PVDF blotting membranes (Millipore, Bedford, MA). For detection of protein expression, membranes were probed with rabbit polyclonal anti-VEGF-A (1:300 dilution, Santa Cruz Biotechnology, CA) and VEGF-C antibody (1:200 dilution), overnight at 4 °C. Recombinant VEGF-A (R & D Systems, Minneapolis, MN) and homogenates of RAW 264.7 cells (Santa Cruz Biotechnology, CA) were used as positive controls for VEGF-A and VEGF-C, respectively. After incubation for 1 h at room temperature with the corresponding secondary antibody tagged with horseradish peroxidase, signals were detected by the chemiluminescence system (Amersham Pharmacia Biotech, Cleveland, OH).

Measurement of VEGF-A and -C secretion from polarized ARPE monolayers: Experiments on VEGF-A and VEGF-C secretion were conducted in transwell filters with confluent ARPE-19 cell monolayers. The filters were incubated either with medium (DMEM/F12) alone or with varying doses of tBH (50±200 µM) for 5 h. Time dependency experiments were performed with 150 µM tBH over 5 h in culture medium. All experiments were performed in duplicate simultaneously and each experiment was repeated 3-4 times on different days. At the end of the experiment, medium from the apical and basolateral compartments were collected, and concentrated through centrifugal filter devices (Millipore) to a final volume of 50 µl and stored at -80 °C until further analysis. In initial studies the effect of tBH on gene and protein expression of VEGF-A and VEGF-C was examined using confluent, nonpolarized ARPE-19 cells in six well plates. Secretion studies were also carried out in confluent, nonpolarized ARPE-19 cells in six well plates treated with varying doses of tBH.

VEGF-A and VEGF-C release to the apical and basolateral sides of the differentiated ARPE-19 cells and VEGF concentration from nonpolarized ARPE-19 cell lysates were assayed by enzyme-linked immunosorbant assay (ELISA; R&D Systems, Minneapolis, MN for VEGF-A; IBL Co., Takasaki-Shi, Japan for VEGF-C), according to the manufacturer's instructions. Data derived from the standard curves were expressed as pg/10⁶ cells.

Data analysis: All experiments were performed in duplicate simultaneously and each experiment was repeated 3-4 times on different days and average±SEM was calculated. Data were analyzed using ANOVA followed by t-test. p<0.05 was considered significant.

RESULTS

Characterization of ARPE-19 cells on transwell filters: The development of a polarized ARPE-19 monolayer was established by immunofluorescent localization of ZO-1 in cultures of one month and older. As shown in Figure 1A, the intercellular assemblage outlining each cell was positively stained for ZO-1. To determine whether oxidative stress af-

fects the integrity of tight junctions, we treated cultures with 150 μM tBH for 5 h and stained for ZO-1. We did not observe any noticeable change in the ZO-1 staining patterns as compared to the untreated controls (Figure 1B).

To further establish that our system is a monolayer and exhibits polarity, we stained also for the tight junction-forming protein occludin as well as the apical marker enzyme Na/K ATPase. As expected, we observed intact staining of the tight junction protein occludin, similar to ZO-1 staining pattern (Figure 2A). As evident from Figure 2B, Na/K ATPase was localized to the apical plasma membrane of the ARPE-19 cells as shown in the confocal vertical (X-Z) section (Figure 2C).

Paracellular permeability: To establish barrier function and to ensure discontinuity between apical and basolateral compartments, we used radiolabeled mannitol to measure paracellular diffusion across the monolayer. These studies

showed that the Papp of mannitol from A-B was $0.88 \pm 0.05 \mu\text{l}/\text{cm}^2/\text{min}$. The Papp from B-A was $0.80 \pm 0.04 \mu\text{l}/\text{cm}^2/\text{min}$, and the values of Papp compare well with 0.25 and 0.5 $\mu\text{l}/\text{cm}^2/\text{min}$ reported by others [31,32]. There was no significant difference in the paracellular permeability of mannitol from the two membrane domains.

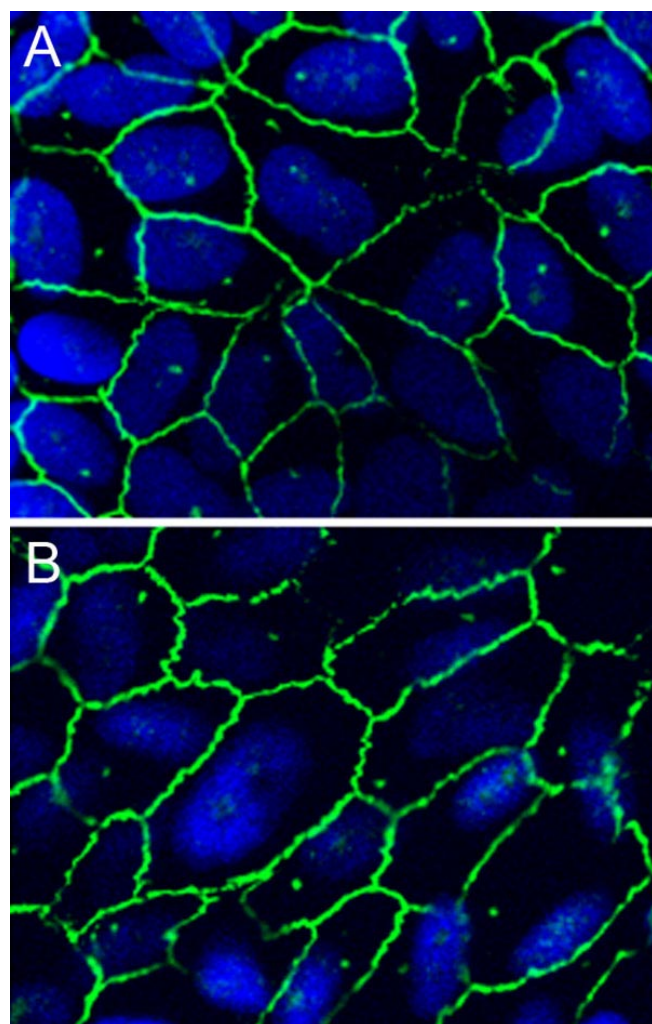


Figure 1. Expression of ZO-1 in polarized ARPE-19 cells grown in transwell filters in 1% fetal bovine serum containing medium. Cells were fixed in 2% paraformaldehyde, blocked in normal goat serum, and incubated with anti-zonula occludens (ZO-1) antibody overnight at 4 °C and mounted with DAPI. **A:** Control, untreated cells **B:** Cells treated with 150 μM t-butylhydroperoxide for 5 h.

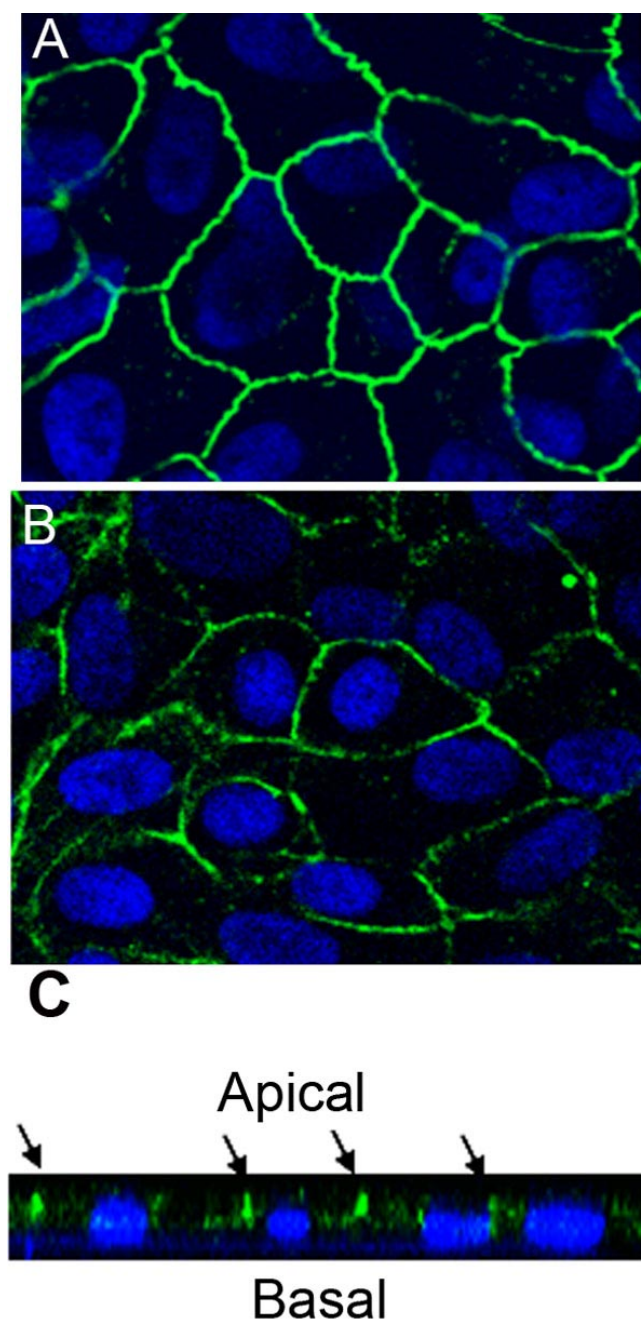


Figure 2. Evidence for tight junctions and polarity in ARPE-19 cells cultured on transwell filters for four weeks. Details of staining procedure are provided in Methods. **A:** Immunofluorescence of tight junctions visualized by occludin staining in ARPE-19 cells. **B:** Immunofluorescence with Na/K ATPase showed that it was mainly localized on the apical plasma membrane of the cells. **C:** Confocal vertical (X-Z) section of Na/K⁺ ATPase staining showing apical localization.

Effect of tBH on tight junction integrity: Weekly measurements of TER were made in cultured ARPE-19 cells maintained in 1% FBS up to one month. The resistance showed a gradual increase with time and began to plateau in one month. The TER values in ARPE-19 monolayers, maintained for one month in 1% FBS-containing medium, averaged $47.5 \pm 4.3 \Omega \cdot \text{cm}^2$ (mean $\pm$ SEM, $n=60$). The TER measurements were also made in ARPE-19 cells exposed to $150 \mu\text{M}$ tBH. The data on the effect of tBH treatment on TER as a function of time for up to 5 h is shown in Figure 3. Exposure of ARPE-19 in transwell filters to $150 \mu\text{M}$ tBH did not cause any significant difference ($p>0.05$) in TER versus untreated controls for any of the time points (Figure 3). The values in ARPE-19 cells after 5 h tBH treatment showed a slight decrease ($<10\%$) at 5 h as compared to untreated controls.

VEGF expression in nonpolarized ARPE-19 cells and effect of tBH: Figure 4 shows the effect of $150 \mu\text{M}$ tBH on VEGF-A mRNA (Figure 4A) and protein in cellular homogenates (Figure 4B) as a function of time. As compared to controls, tBH caused a linear increase in gene expression with the highest significant increase (about eight fold) at 5 h. VEGF-A protein expression also showed a significant increase with tBH treatment at 4 and 5 h, which was 2 and 2.4 fold higher ($p<0.05$ versus untreated controls), respectively (Figure 4B).

The corresponding changes in VEGF-C mRNA and protein are shown in Figure 5A,B. Unlike VEGF-A, levels of VEGF-C mRNA after tBH treatment were not significantly higher than controls except at 5 h at which time the increase

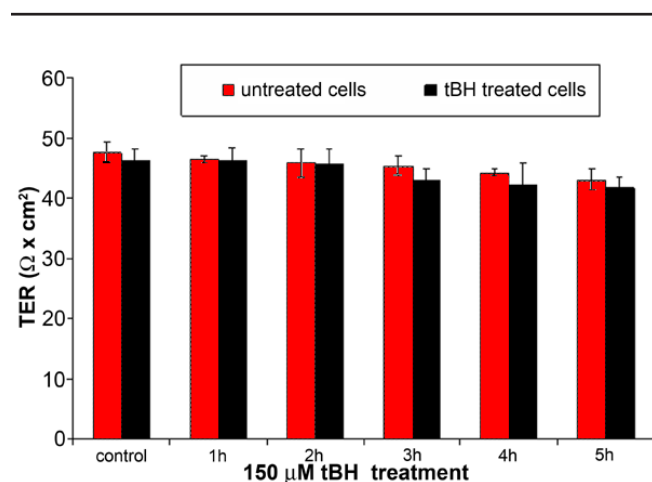


Figure 3. Effect of t-butylhydroperoxide treatment on transepithelial resistance in polarized ARPE-19 monolayers. ARPE-19 cells were treated with $150 \mu\text{M}$ tBH for varying times up to 5 h and TER was measured at indicated time points in t-butylhydroperoxide-treated ARPE and non-treated controls. Data are mean $\pm$ SEM ($n=4$). The TER values corrected for blank showed a slight decline with increasing time of exposure in the untreated control and tBH group, which was not statistically significant compared to the corresponding controls. The grand mean TER from a number of experiments was $47.5 \pm 4.3 \Omega \cdot \text{cm}^2$ ($n=60$) in untreated controls and $41.5 \pm 1.7 \Omega \cdot \text{cm}^2$ ($n=40$) from tBH-treated ARPE cells.

was 1.8 fold as that of control (Figure 5A). The VEGF-C protein level showed a time-dependent increase, and this increase was 1.5 and 2.1 fold higher than controls ($p<0.05$) at 4 h and 5 h of tBH treatment, respectively (Figure 5B).

In separate experiments, we also determined VEGF-A concentration in nonpolarized ARPE-19 cells and the effect of $150 \mu\text{M}$ tBH treatment as a function of time using ELISA analysis. Similar to the western blot analysis data, the cellular concentration of VEGF-A increased with time of tBH exposure. The mean VEGF-A concentrations (pg/ 10^6 cells) by ELISA analysis were 16.5 ± 1.7 (untreated control), (1 h) 27.8 ± 2.7 , (2 h) 62.2 ± 9.3 , (3 h) 69.7 ± 6.9 , (4 h) 128 ± 19.2 , and (5 h) 168.6 ± 25.2 , respectively. Thus an approximately ten fold increase in VEGF-A levels were found at 5 h tBH treatment when compared to untreated, zero time controls.

VEGF secretion in nonpolarized ARPE-19 cells and effect of tBH: Release of VEGF-A and VEGF-C from non-polarized, confluent ARPE-19 cells and the effect of tBH was determined in initial experiments. Figure 6 shows the secretion in pg/ 10^6 cells of VEGF-A and C after 5 h treatment with tBH. Untreated RPE cells secreted 84 ± 24 pg/ 10^6 cells and 117 ± 24 pg/ 10^6 cells of VEGF-A and VEGF-C, respectively. tBH treatment caused a significant ($p<0.05$ versus control) increase in VEGF-A secretion at 5 h (Figure 6A). On the other hand, tBH treatment caused a slight increase in VEGF-C secretion up to 5 h but the increase was not significantly different from that of untreated controls (Figure 6B).

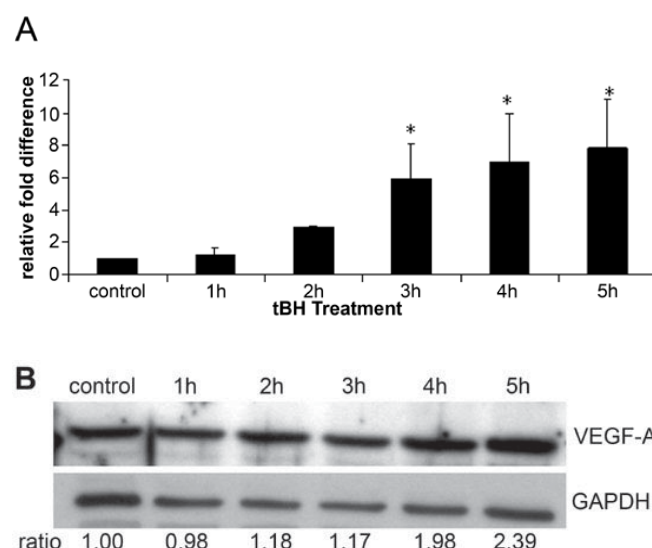


Figure 4. Effect of t-butylhydroperoxide on vascular endothelial growth factor-A gene and protein expression in ARPE-19 cells. Effect of t-butylhydroperoxide treatment on expression of vascular endothelial growth factor-A (VEGF-A) mRNA (A) and protein (B) in confluent, nonpolarized ARPE-19 cells. The mRNA and protein were analyzed by realtime PCR and immunoblot analysis, respectively, the latter under reducing condition. Data are mean $\pm$ SEM from three experiments. tBH treatment caused a linear increase in gene expression with a maximum increase at 5 h. Asterisk indicates $p<0.05$ versus control. Recombinant VEGF-A was used as a positive control to identify the VEGF-A band in the western blot.

Effect of tBH on VEGF-A in polarized ARPE-19 cells:

The amounts of VEGF-A and VEGF-C (data not shown) secreted by untreated polarized ARPE-19 cells (Figure 7) were 5 and 9 fold higher ($p<0.01$) than the secretion by untreated non-polarized ARPE-19 cells grown to confluence (see Figure 6), suggesting that polarization stimulates secretion in RPE. Both VEGF-A and C were secreted from apical and basolateral surfaces. However, secretion from the apical side was significantly ($p<0.01$) higher than that from the basolateral domain at 50 and 100 μM tBH treatment. Figure 7 shows the dose-dependent increase in secretion of VEGF-A with tBH in ARPE-19 transwell filters. Doses in the range of 50 μM -200 μM were used in these experiments and the exposure time was 5 h. The baseline secretion of VEGF-A in confluent untreated control RPE cells was 870 pg/ 10^6 cells for apical and 602 pg/ 10^6 cells for the basolateral sides. The secretion increased with dose of tBH for both apical and basolateral sides with a maximal increase of 63% and 125%, respectively (Figure 7). The mean secretion of VEGF-A to the apical side was larger than basolateral secretion for the dose range 50-150 μM and the apical secretory rate for 200 μM tBH. We used a constant dose of 150 μM tBH in our experiments to examine the time de-

pendency of secretion, and these data are presented in Figure 8. In these studies, the basolateral secretion of VEGF-A was linear, although the absolute levels of secretion tended to be lower than that of apical VEGF-A secretion during the entire time course. Figure 8 also shows the rates of secretion in the apical and basolateral direction of ARPE cells, which were not treated with tBH. While VEGF-A secretion was found in both apical and basolateral sides in untreated cells, this secretion was much lower than that with tBH treatment. VEGF-A secretion in untreated cells did not exceed 12% of the secretion to apical and 17% of secretion to basolateral domain of tBH-treated cells at 5 h (Figure 8).

Effect of tBH on VEGF-C secretion in polarized ARPE-19 cells: Based on data derived from the aforescribed secretion studies with VEGF-A, we chose a dose of 150 μM tBH for 5 h duration in time-course studies of VEGF-C secretion. As found for VEGF-A secretion, 150 μM tBH caused a

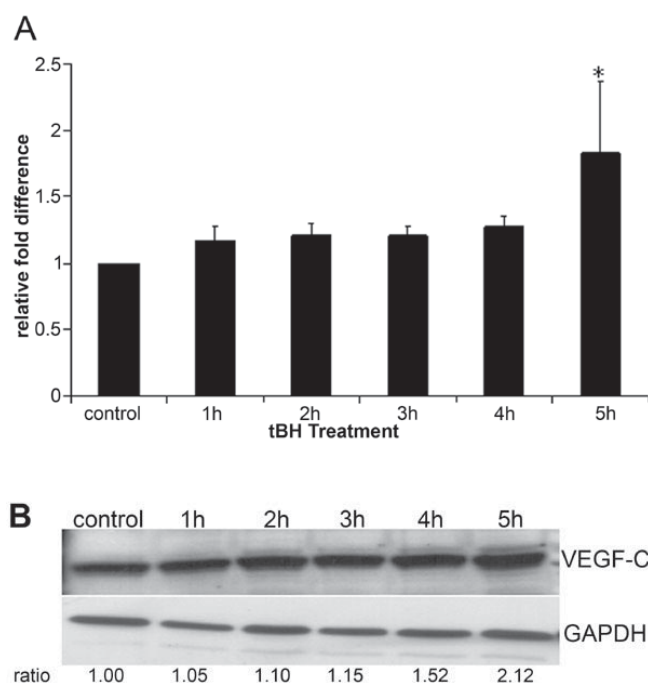


Figure 5. Effect of t-butylhydroperoxide on vascular endothelial growth factor-C gene and protein expression in ARPE-19 cells. Effect of t-butylhydroperoxide treatment on vascular endothelial growth factor-C (VEGF-C) mRNA (**A**) and protein (**B**) expression in confluent non-polarized ARPE-19 cells. Data are mean $\pm$ SEM from three experiments. Experimental conditions are the same as in Figure 4. At the 5 h time point, mRNA for t-butylhydroperoxide treated cells shows a significant 1.87 fold increase over the control. Asterisk indicates $p<0.05$ versus control. RAW 264.7 cells were used as a positive control to identify the VEGF-C band in the western blot analysis in Figure 4B.

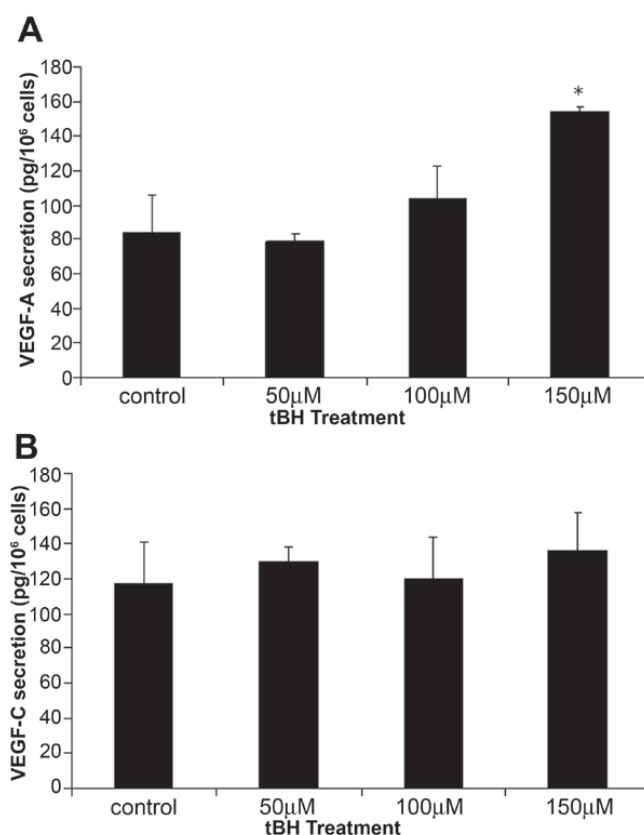


Figure 6. Effect of t-butylhydroperoxide on vascular endothelial growth factor-A and vascular endothelial growth factor-C secretion from ARPE-19 cells. **A:** Secretion of vascular endothelial growth factor-A (VEGF-A) in confluent control ARPE-19 cells and cells treated with varying doses of tBH for 5 h. Asterisk indicates VEGF-A secretion at 5 h with 150 μM t-butylhydroperoxide (tBH) was significantly ($p<0.05$) higher than the untreated controls. Data are mean $\pm$ SEM ($n=4$ /time point in each group). **B:** Secretion of VEGF-C in confluent control ARPE-19 cells and cells treated with varying doses of tBH for 5 h. VEGF-C secretion after 5 h tBH treatment with varying doses was not significantly different from the untreated controls. Data are mean $\pm$ SEM ($n=4$ /time point in each group).

time-dependent increase in apical and basolateral VEGF-C secretion with the highest secretion found at 5 h (Figure 9). However, the total amount of VEGF-C secreted by ARPE-19 cells was significantly lower than that of VEGF-A (see Figure 8). Furthermore, the secretion at 5 h for the basolateral side was significantly lower than that of apical secretion. We could not accurately determine the secretory rate of VEGF-C in untreated polarized ARPE-19 monolayer because of the low levels and limitation of sensitivity of our ELISA assay for VEGF-C measurement.

DISCUSSION

We have studied the effect of oxidative stress on the secretion of A and C isoforms of VEGF in ARPE-19 cells. The ARPE-19 monolayers in transwell filters exhibited a highly polarized phenotype as evidenced by their TER, ZO-1 and occludin expression and apical localization of Na/K ATPase. In the polarized ARPE-19 model, VEGF-A secretion was prominently stimulated on exposure to tBH. The increase in secretion was higher on the apical than the basolateral side for VEGF-A and -C. Our study also showed for the first time, that VEGF-C secretion was upregulated by oxidative stress in a time-dependent fashion.

The functional validity of the cultured cell model depends on its potential to exhibit *in vivo* features. Our data revealed some of the functional properties exhibited by *in vivo* systems, such as ZO-1 and occludin staining at the tight junctions and apical localization of Na/K ATPase, were found in the ARPE-19 cells and were consistent with previous findings [31-33]. These features correlated with decreased paracellular permeability of mannitol in our study as in previous studies in which paracellular permeability decreased with the maturation of RPE [33,34].

In our study, ZO-1 expression pattern remained unaltered with tBH treatment of polarized cells. Exposure of ARPE-19 cells to 0.5 or 2 mM H_2O_2 for 24 h was shown to cause an increase in transepithelial flux across the RPE monolayer from disruption of tight junction proteins [33]. In another study,

paracellular changes by H_2O_2 in porcine-brain-derived endothelial cells correlated with changes in the junction proteins [35]. The discrepancy in results between the earlier studies and our present study may arise from the high doses of oxidative agents used or prolonged treatment period or difference in cell types in earlier reports. We used less severe stress and for a short time-conditions that did not significantly affect cell viability. In favor of this view, Alizadeh et al. [36] reported that polarized ARPE cells were viable at different tBH concentrations (50 μ M-3 mM) treatment for 30 min, whereas H_2O_2 (50 μ M-3 mM) treatment caused significant loss of cell viability at doses higher than 300 μ M.

VEGF is implicated as a pro-angiogenic factor stimulating neovascularization in AMD and an important source is likely to be the RPE [37]. VEGF expression by RPE is a feature of tissues excised from human eyes showing AMD-related CNV [7,38]. The relationship between VEGF and CNV is also exemplified by studies showing that overproduction of VEGF by RPE cells, as a result of adenovirus-mediated transduction or expression in transgenic mice, leads to new blood vessel growth [39,40]. VEGF has been implicated as a mediator of intraocular neovascularization and its expression is temporally and spatially correlated with the development of new preretinal vessels [41]. While most studies on VEGF refer to VEGF-A isoform, information on VEGF-C expression and to role in the retina is scarce. One isolated report suggests that following 24 h hypoxia, VEGF-C increased 3 fold in the mouse retina, suggesting a role in retinal ischemia [42]. VEGF-C induces angiogenesis in the context of tissue ischemia [43]. Recently, Ikeda et al. [21] detected VEGF-C and VEGF-D proteins in the aqueous humor and vitreous fluid of patients, suggesting a possible role for them in ocular angiogenesis.

Oxidant injury is a potent stimulus for VEGF expression [44]. A decrease in cellular antioxidant ability or increase in oxidative insults can induce oxidative stress. We found treatment of confluent, but nonpolarized, ARPE-19 cells with dif-

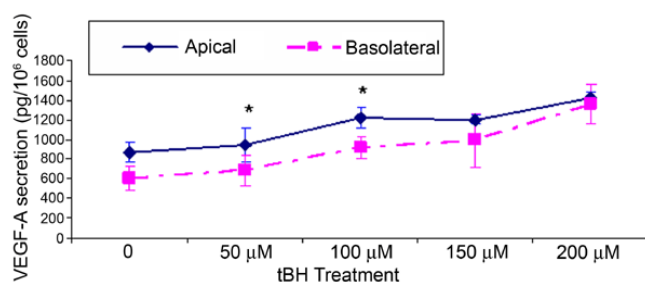


Figure 7. Dose-dependent effect of t-butylhydroperoxide treatment on apical and basolateral secretion of vascular endothelial growth factor-A in polarized ARPE-19 monolayers. Secretion at 5 h treatment with t-butylhydroperoxide of vascular endothelial growth factor-A (VEGF-A) was significantly higher ($p < 0.05$) versus the untreated controls. Processing of cells for VEGF secretion and calculations are given in Methods. Data are mean $\pm$ SEM ($n = 4$).

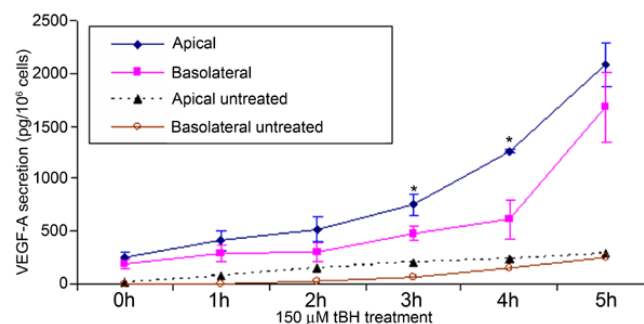


Figure 8. Time-dependent secretion of vascular endothelial growth factor-A in polarized ARPE-19 monolayers after 150 μ M t-butylhydroperoxide treatment. Apical and basolateral vascular endothelial growth factor-A (VEGF-A) secretion increased as a function of time of tBH treatment with maximal increase at 5 h ($p < 0.05$ versus controls). Figure also shows VEGF-A secretion in untreated controls on the apical (dotted line) and basolateral (open circles). Data are mean $\pm$ SEM from 3-4-determinations/time point.

ferent doses of tBH increased VEGF-A significantly over control untreated cells. This data was similar to previous findings in ARPE-19 treated with 4-hydroxynonenal [45]. Stimulation of VEGF-A secretion with oxidative stress, hypoxia, and in diseases such as diabetic retinopathy and AMD [41,46,47] has also been reported. We recently reported that in human RPE cells oxidative stress caused by glutathione depletion stimulated VEGF secretion and increased expression of its receptors [48].

Differentiated ARPE cells produce more ROS when treated with tBH than H_2O_2 [36]. It is likely that ROS generated from tBH exposure may contribute to increased VEGF secretion although whether tBH and ROS activate other factors is not fully understood. A recent study revealed that H_2O_2 treatment enhances VEGF through upregulation of hypoxia-inducible factor-1 (HIF-1) expression [49]. Furthermore, cytokines, such as insulin-like growth factor-1 (IGF-1), are also known to stimulate VEGF secretion via HIF-1-dependent and -independent pathways [50]. Data on VEGF synthesis and secretion in the present study were collected from RPE cells after short term (eg 5 h) exposure to tBH. The ten fold increase in intracellular expression of VEGF found by ELISA at 5 h suggested that evaluation of later time points would show even greater levels of VEGF secretion. Such studies would need to be performed at lower tBH concentrations to avoid cytotoxicity. Additional work will be needed to evaluate the effects of chronic oxidant exposure and to delineate the specific pathways involved in tBH-induced VEGF secretion in our model system [51].

Studies have previously demonstrated that cultured human RPE cells secrete VEGF [52,53]. The RPE is a major source of angiogenic and antiangiogenic factors, and dysregulation of the balance between them contributes to the modulation and progression of retinal vascular disease, including CNV [54,55]. The RPE and surrounding subretinal membranes of CNV-afflicted retinas express high levels of VEGF and its receptor [56,57]. Although rather different factors regu-

late VEGF production [54], the present study revealed that acute oxidant injury also induced its production in polarized ARPE-19 cells. Our study showed that polarized ARPE-19 cells secrete VEGF-A and C preferentially to their apical (retina facing) side, observations in agreement with Marmorstein et al. [58] in RPE-J cells and Slomiany and Rosenzweig [59] in D407 cells. On the contrary, it has also been reported that VEGF-A secretion is mainly basolateral and increases 1.3-1.5 fold during hypoxic conditions [60]. This disparity in results could be due to the type of cell lines used or due to simple diffusion from the basolateral compartment to apical side, which is likely to occur as a result of the difference in medium volume. In contrast, the high apical secretion of VEGF-A and C in the present study using ARPE-19 and in previous studies [58,59] resulted in VEGF accumulating against the diffusion gradient. The preferentially apical secretion of VEGF by RPE cells into the subretinal space though at first seems at odds with the progression of CNV, given that the tight junctional barrier should inhibit the diffusion of VEGF to the choroid. However, apical secretion of VEGF would promote a VEGF gradient that is highest in the subretinal space thereby promoting the growth of CNV through breaks in Bruch's membrane and the RPE monolayer into the subretinal space to form classic CNV. Increased apical secretion may also provide a beneficial neuroprotective effect; VEGF has been shown to possess neurotrophic, neuroprotective, as well as angiogenic properties [61]. It was suggested that apically secreted VEGF provides the driving signal for CNV development [59]. As a support to this view, CNV was found to develop in a mouse transgenic model where VEGF was overexpressed in RPE cells [39]. Spilisbury et al. [40] found that recombinant adenoviral gene delivery of VEGF, specifically targeting the RPE, led to CNV in the rat. It has been reported that the slow release of bioactive VEGF protein from pellets implanted between the choroid and sclera do not result in CNV [62] or vascular leakage, suggesting that the development of CNV requires VEGF to be secreted from the RPE toward the inner choroid in which high levels of VEGF receptors have been found. Since VEGF may induce changes in tight junction structure and function in RPE, paracellular diffusion of VEGF toward the choroid may also contribute to neovascularization. This may explain the lower basolateral VEGF secretion that was observed. Low basolateral levels of VEGF may be required for physiological maintenance of the choriocapillaris.

Our results also demonstrated that VEGF-C is expressed in ARPE-19 cells, and oxidative stress induced by tBH stimulates VEGF-C secretion in nonpolarized RPE. Further, our studies using ARPE-19 in transwell filters showed for the first time that VEGF-C is secreted to both apical and basolateral sides. Treatment with tBH stimulated secretion of VEGF-C to apical and basolateral sides, the secretion to apical side being higher than that of basolateral. An interesting postulate has been proposed recently in which VEGF-A has an important role in the regulation of VEGF-C expression in the RPE and the interaction of VEGF-A with VEGF-C was suggested to play a role in the pathology of CNV [63].

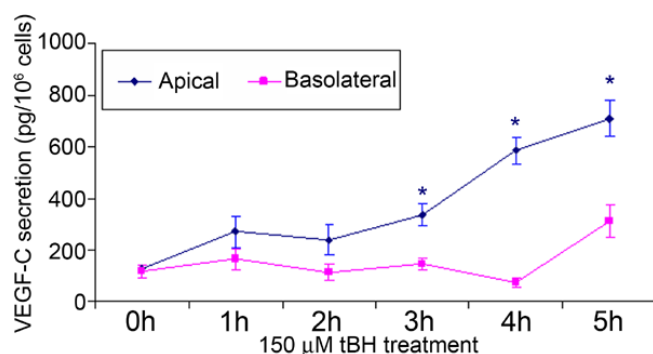


Figure 9. Time-dependent apical and basolateral vascular endothelial growth factor-C secretion by polarized ARPE-19 cells treated with 150 μ M t-butylhydroperoxide for 5 h. Data are mean $\pm$ SEM (n=4/time point). Vascular endothelial growth factor-C (VEGF-C) secretion in basolateral side was significantly lower ($p < 0.05$) as compared to vascular endothelial growth factor-A secretion.

In conclusion, we have shown that oxidative stress causes increased secretion of VEGF-A and VEGF-C in ARPE-19 cells with higher secretion to the apical domain as compared to the basolateral domain. The significance of this finding with relation to devising therapeutic strategies for preventing neovascularization remains to be further explored.

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REFERENCES

- Cai J, Nelson KC, Wu M, Sternberg P Jr, Jones DP. Oxidative damage and protection of the RPE. *Prog Retin Eye Res* 2000; 19:205-21.
- Thumann G, Hinton DR. Cell biology of the retinal pigment epithelium. In: Ryan SJ, editor. *Retina*. 3rd ed. Vol 1. St. Louis: Mosby; 2001. p.104-21.
- Sakamoto T, Sakamoto H, Hinton DR, Spee C, Ishibashi T, Ryan SJ. In vitro studies of human choroidal endothelial cells. *Curr Eye Res* 1995; 14:621-7.
- Miller H, Miller B, Ryan SJ. The role of retinal pigment epithelium in the involution of subretinal neovascularization. *Invest Ophthalmol Vis Sci* 1986; 27:1644-52.
- Ellis EA, Grant MB, Murray FT, Wachowski MB, Guberski DL, Kubilis PS, Luty GA. Increased NADH oxidase activity in the retina of the BBZ/Wor diabetic rat. *Free Radic Biol Med* 1998; 24:111-20.
- Ellis EA, Guberski DL, Somogyi-Mann M, Grant MB. Increased H₂O₂, vascular endothelial growth factor and receptors in the retina of the BBZ/Wor diabetic rat. *Free Radic Biol Med* 2000; 28:91-101.
- Lopez PF, Sippy BD, Lambert HM, Thach AB, Hinton DR. Transdifferentiated retinal pigment epithelial cells are immunoreactive for vascular endothelial growth factor in surgically excised age-related macular degeneration-related choroidal neovascular membranes. *Invest Ophthalmol Vis Sci* 1996; 37:855-68.
- Higgins RD, Sanders RJ, Yan Y, Zasloff M, Williams JI. Squalamine improves retinal neovascularization. *Invest Ophthalmol Vis Sci* 2000; 41:1507-12.
- Witmer AN, Vrensen GF, Van Noorden CJ, Schlingemann RO. Vascular endothelial growth factors and angiogenesis in eye disease. *Prog Retin Eye Res* 2003; 22:1-29.
- Veikkola T, Karkkainen M, Claesson-Welsh L, Alitalo K. Regulation of angiogenesis via vascular endothelial growth factor receptors. *Cancer Res* 2000; 60:203-12.
- Witmer AN, Dai J, Weich HA, Vrensen GF, Schlingemann RO. Expression of vascular endothelial growth factor receptors 1, 2, and 3 in quiescent endothelia. *J Histochem Cytochem* 2002; 50:767-77.
- Kim I, Ryan AM, Rohan R, Amano S, Agular S, Miller JW, Adamis AP. Constitutive expression of VEGF, VEGFR-1, and VEGFR-2 in normal eyes. *Invest Ophthalmol Vis Sci* 1999; 40:2115-21. Erratum in: *Invest Ophthalmol Vis Sci* 2000 Feb;41(2):368.
- Ladoux A, Frelin C. Hypoxia is a strong inducer of vascular endothelial growth factor mRNA expression in the heart. *Biochem Biophys Res Commun* 1993; 195:1005-10.
- Chua CC, Hamdy RC, Chua BH. Upregulation of vascular endothelial growth factor by H₂O₂ in rat heart endothelial cells. *Free Radic Biol Med* 1998; 25:891-7.
- Hoffmann S, Friedrichs U, Eichler W, Rosenthal A, Wiedemann P. Advanced glycation end products induce choroidal endothelial cell proliferation, matrix metalloproteinase-2 and VEGF upregulation in vitro. *Graefes Arch Clin Exp Ophthalmol* 2002; 240:996-1002.
- Naginei CN, Samuel W, Naginei S, Pardhasaradhi K, Wiggert B, Detrick B, Hooks JJ. Transforming growth factor-beta induces expression of vascular endothelial growth factor in human retinal pigment epithelial cells: involvement of mitogen-activated protein kinases. *J Cell Physiol* 2003; 197:453-62.
- Masood R, Cai J, Zheng T, Smith DL, Hinton DR, Gill PS. Vascular endothelial growth factor (VEGF) is an autocrine growth factor for VEGF receptor-positive human tumors. *Blood* 2001; 98:1904-13.
- Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, Saksela O, Kalkkinen N, Alitalo K. A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J* 1996; 15:290-98. Erratum in: *EMBO J*. 1996 Apr 1;15(7):1751.
- Makinen T, Veikkola T, Mustjoki S, Karpanen T, Catimel B, Nice EC, Wise L, Mercer A, Kowalski H, Kerjaschki D, Stacker SA, Achen MG, Alitalo K. Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3. *EMBO J* 2001; 20:4762-73.
- Veikkola T, Jussila L, Makinen T, Karpanen T, Jeltsch M, Petrova TV, Kubo H, Thurston G, McDonald DM, Achen MG, Stacker SA, Alitalo K. Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. *EMBO J* 2001; 20:1223-31.
- Ikeda Y, Yonemitsu Y, Onimaru M, Nakano T, Miyazaki M, Kohno R, Nakagawa K, Ueno A, Sueishi K, Ishibashi T. The regulation of vascular endothelial growth factors (VEGF-A, -C, and -D) expression in the retinal pigment epithelium. *Exp Eye Res* 2006; 83:1031-40.
- Rodriguez-Boulton E, Nelson WJ. Morphogenesis of the polarized epithelial cell phenotype. *Science* 1989; 245:718-25.
- Tsukita S, Furuse M, Itoh M. Multifunctional strands in tight junctions. *Nat Rev Mol Cell Biol* 2001; 2:285-93.
- Powell DW. Barrier function of epithelia. *Am J Physiol* 1981; 241:G275-88.
- Stevenson BR, Siliciano JD, Mooseker MS, Goodenough DA. Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. *J Cell Biol* 1986; 103:755-66.
- Dunn KC, Aotaki-Keen AE, Putkey FR, Hjelmeland LM. ARPE-19, a human retinal pigment epithelial cell line with differentiated properties. *Exp Eye Res* 1996; 62:155-69.
- Holtkamp GM, Van Rossem M, de Vos AF, Willekens B, Peek R, Kijlstra A. Polarized secretion of IL-6 and IL-8 by human retinal pigment epithelial cells. *Clin Exp Immunol* 1998; 112:34-43.
- Narayan S, Prasanna G, Krishnamoorthy RR, Zhang X, Yorio T. Endothelin-1 synthesis and secretion in human retinal pigment epithelial cells (ARPE-19): differential regulation by cholinergics and TNF-alpha. *Invest Ophthalmol Vis Sci* 2003; 44:4885-94.
- Abe T, Sugano E, Saigo Y, Tamai M. Interleukin-1beta and barrier function of retinal pigment epithelial cells (ARPE-19): aberrant expression of junctional complex molecules. *Invest Ophthalmol Vis Sci* 2003; 44:4097-104.

30. Zhang N, Kannan R, Okamoto CT, Ryan SJ, Lee VH, Hinton DR. Characterization of brimonidine transport in retinal pigment epithelium. *Invest Ophthalmol Vis Sci* 2006; 47:287-94.
31. Ban Y, Rizzolo LJ. A culture model of development reveals multiple properties of RPE tight junctions. *Mol Vis* 1997; 3:18.
32. Rajasekaran SA, Hu J, Gopal J, Gallemore R, Ryazantsev S, Bok D, Rajasekaran AK. Na,K-ATPase inhibition alters tight junction structure and permeability in human retinal pigment epithelial cells. *Am J Physiol Cell Physiol* 2003; 284:C1497-507.
33. Bailey TA, Kanuga N, Romero IA, Greenwood J, Luthert PJ, Cheetham ME. Oxidative stress affects the junctional integrity of retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci* 2004; 45:675-84.
34. Williams CD, Rizzolo LJ. Remodeling of junctional complexes during the development of the outer blood-retinal barrier. *Anat Rec* 1997; 249:380-8.
35. Fischer S, Wiesnet M, Renz D, Schaper W. H₂O₂ induces paracellular permeability of porcine brain-derived microvascular endothelial cells by activation of the p44/42 MAP kinase pathway. *Eur J Cell Biol* 2005; 84:687-97.
36. Alizadeh M, Wada M, Gelfman CM, Handa JT, Hjelmeland LM. Downregulation of differentiation specific gene expression by oxidative stress in ARPE-19 cells. *Invest Ophthalmol Vis Sci* 2001; 42:2706-13.
37. Kliffen M, Sharma HS, Mooy CM, Kerkvliet S, de Jong PT. Increased expression of angiogenic growth factors in age-related maculopathy. *Br J Ophthalmol* 1997; 81:154-62.
38. Grossniklaus HE, Ling JX, Wallace TM, Dithmar S, Lawson DH, Cohen C, Elner VM, Elner SG, Sternberg P Jr. Macrophage and retinal pigment epithelium expression of angiogenic cytokines in choroidal neovascularization. *Mol Vis* 2002; 8:119-26.
39. Schwesinger C, Yee C, Rohan RM, Jousen AM, Fernandez A, Meyer TN, Poulaki V, Ma JJ, Redmond TM, Liu S, Adamis AP, D'Amato RJ. Intrachoroidal neovascularization in transgenic mice overexpressing vascular endothelial growth factor in the retinal pigment epithelium. *Am J Pathol* 2001; 158:1161-72.
40. Spilisbury K, Garrett KL, Shen WY, Constable IJ, Rakoczy PE. Overexpression of vascular endothelial growth factor (VEGF) in the retinal pigment epithelium leads to the development of choroidal neovascularization. *Am J Pathol* 2000; 157:135-44. Erratum in: *Am J Pathol* 2000 Oct;157(4):1413.
41. Aiello LP, Avery RL, Arrigg PG, Keyt BA, Jampel HD, Shah ST, Pasquale LR, Thieme H, Iwamoto MA, Park JE, Nguyen HV, Aiello LM, Ferrara N, King GL. Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N Engl J Med* 1994; 331:1480-7.
42. Simpson DA, Murphy GM, Bhaduri T, Gardiner TA, Archer DB, Stitt AW. Expression of the VEGF gene family during retinal vaso-obliteration and hypoxia. *Biochem Biophys Res Commun* 1999; 262:333-40.
43. Witzienbichler B, Asahara T, Murohara T, Silver M, Spyridopoulos I, Magner M, Principe N, Kearney M, Hu JS, Isner JM. Vascular endothelial growth factor-C (VEGF-C/VEGF-2) promotes angiogenesis in the setting of tissue ischemia. *Am J Pathol* 1998; 153:381-94.
44. Brown NS, Jones A, Fujiyama C, Harris AL, Bicknell R. Thymidine phosphorylase induces carcinoma cell oxidative stress and promotes secretion of angiogenic factors. *Cancer Res* 2000; 60:6298-302.
45. Ayalasomayajula SP, Kompella UB. Induction of vascular endothelial growth factor by 4-hydroxynonenal and its prevention by glutathione precursors in retinal pigment epithelial cells. *Eur J Pharmacol* 2002; 449:213-20.
46. Mathews MK, Merges C, McLeod DS, Luttly GA. Vascular endothelial growth factor and vascular permeability changes in human diabetic retinopathy. *Invest Ophthalmol Vis Sci* 1997; 38:2729-41.
47. Mousa SA, Lorelli W, Campochiaro PA. Role of hypoxia and extracellular matrix-integrin binding in the modulation of angiogenic growth factors secretion by retinal pigmented epithelial cells. *J Cell Biochem* 1999; 74:135-43.
48. Sreekumar PG, Kannan R, de Silva AT, Burton R, Ryan SJ, Hinton DR. Thiol regulation of vascular endothelial growth factor-A and its receptors in human retinal pigment epithelial cells. *Biochem Biophys Res Commun* 2006; 346:1200-6.
49. Lee KS, Kim SR, Park SJ, Park HS, Min KH, Lee MH, Jin SM, Jin GY, Yoo WH, Lee YC. Hydrogen peroxide induces vascular permeability via regulation of vascular endothelial growth factor. *Am J Respir Cell Mol Biol* 2006; 35:190-7.
50. Slomiany MG, Rosenzweig SA. Hypoxia-inducible factor-1-dependent and -independent regulation of insulin-like growth factor-1-stimulated vascular endothelial growth factor secretion. *J Pharmacol Exp Ther* 2006; 318:666-75.
51. Slomiany MG, Rosenzweig SA. Autocrine effects of IGF-I-induced VEGF and IGFBP-3 secretion in retinal pigment epithelial cell line ARPE-19. *Am J Physiol Cell Physiol* 2004; 287:C746-53.
52. Adamis AP, Shima DT, Yeo KT, Yeo TK, Brown LF, Berse B, D'Amore PA, Folkman J. Synthesis and secretion of vascular permeability factor/vascular endothelial growth factor by human retinal pigment epithelial cells. *Biochem Biophys Res Commun* 1993; 193:631-8.
53. Campochiaro PA, Hackett SF. Ocular neovascularization: a valuable model system. *Oncogene* 2003; 22:6537-48.
54. Punglia RS, Lu M, Hsu J, Kuroki M, Tolentino MJ, Keough K, Levy AP, Levy NS, Goldberg MA, D'Amato RJ, Adamis AP. Regulation of vascular endothelial growth factor expression by insulin-like growth factor I. *Diabetes* 1997; 46:1619-26.
55. Kvant A, Algvere PV, Berglin L, Seregard S. Subfoveal fibrovascular membranes in age-related macular degeneration express vascular endothelial growth factor. *Invest Ophthalmol Vis Sci* 1996; 37:1929-34.
56. Amin R, Puklin JE, Frank RN. Growth factor localization in choroidal neovascular membranes of age-related macular degeneration. *Invest Ophthalmol Vis Sci* 1994; 35:3178-88.
57. Seregard S, Algvere PV, Berglin L. Immunohistochemical characterization of surgically removed subfoveal fibrovascular membranes. *Graefes Arch Clin Exp Ophthalmol* 1994; 32:325-9.
58. Marmorstein AD, Csaky KG, Baffi J, Lam L, Rahaal F, Rodriguez-Boulan E. Saturation of, and competition for entry into, the apical secretory pathway. *Proc Natl Acad Sci U S A* 2000; 97:3248-53.
59. Slomiany MG, Rosenzweig SA. IGF-1-induced VEGF and IGFBP-3 secretion correlates with increased HIF-1 alpha expression and activity in retinal pigment epithelial cell line D407. *Invest Ophthalmol Vis Sci* 2004; 45:2838-47.
60. Blaauwgeers HG, Holtkamp GM, Rutten H, Witmer AN, Koolwijk P, Partanen TA, Alitalo K, Kroon ME, Kijlstra A, van Hinsbergh VW, Schlingemann RO. Polarized vascular endothelial growth factor secretion by human retinal pigment epithelium and localization of vascular endothelial growth factor receptors on the inner choriocapillaris. Evidence for a trophic paracrine relation. *Am J Pathol* 1999; 155:421-8.
61. Gora-Kupilas K, Josko J. The neuroprotective function of vascular endothelial growth factor (VEGF). *Folia Neuropathol* 2005; 43:31-9.

62. Kim SI, Ng EW, Kenney AG, Tolentino MJ, Connolly EJ, Gragoudas ES, Miller JW. Rat model of subretinal choroidal neovascular membrane formation: preliminary results. *Invest Ophthalmol Vis Sci* 1996; 37:S125
63. Zhao B, Ma A, Cai J, Boulton M. VEGF-A regulates the expression of VEGF-C in human retinal pigment epithelial cells. *Br J Ophthalmol* 2006; 90:1052-9.